Isolation and characterization of a calendic acid producing (8,11)-linoleoyl desaturase[1]

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Abstract **For the biosynthesis of calendic acid a (8,11)-linoleoyl desaturase activity has been proposed. To isolate this desaturase, PCR-based cloning was used. The open reading frame of the isolated full-length cDNA is a 1131 bp sequence encoding a protein of 377 amino acids. For functional identification the cDNA was expressed in *Saccharomyces cerevisiae*, and formation of calendic acid was analyzed by RP-HPLC. The expression of the heterologous enzyme resulted in a significant amount of calendic acid presumably esterified within phospholipids. The results presented here identify a gene encoding a new type of (1,4)-acyl lipid desaturase.**

Key words: Acyl-group desaturase; Seed oil; *Calendula officinalis*; *Saccharomyces cerevisiae*

1. Introduction

The most common octadecatrienoic fatty acid in plants is α-linoleic acid (9*Z*,12*Z*,15*Z*-18:3), which is the main constituent of chloroplastic membranes [1]. Moreover, triacylglycerols from seeds provide an easily accessible source of additional conjugated octadecatrienoic acids having (*Z*,*E*,*E*) or (*Z*,*E*,*Z*) geometries [2]. At least five different regio isomers have been reported within plants with double bond systems in the following positions: (*Z*,*E*,*Z*)- and (*E*,*E*,*Z*)-8,10,12-18:3 and (*Z*,*E*,*Z*)-, (*Z*,*E*,*E*)- and (*E*,*E*,*Z*)-9,11,13-18:3. One of these, calendic acid (8*E*,10*E*,12*Z*-18:3), is the major constituent of the seed oil of *Calendula officinalis*, *Calendula stellata*, *Osteospermum spinescens* and *Osteospermum hyoseroides* [1]. These seed oils are of industrial interest, because the oil is used for cosmetic purposes and may be used as a drying oil in paintings.

A number of chemical mechanisms have been proposed to describe the biosynthesis of conjugated octadecatrienoic acids. These include the formation of an epoxy derivative of linoleic acid as an intermediate [3], the oxidation of linoleic acid [4], the isomerization of linolenic acid [5] and the formation of a radical of linoleic acid due to a lipoxygenase-type reaction [6–8]. However, in a recent publication on the biosynthesis of α-eleostearic acid (9*Z*,11*E*,13*E*-18:3), which is a regio isomer of calendic acid, it was shown that the conversion of linoleic acid to this conjugated octadecatrienoic acid occurs while this acyl moiety is esterified to phosphatidylcholine [9]. Based on these data one may assume that the conversion of esterified linoleic acid to conjugated octadecatrienoic acids is catalyzed by a new type of (1,4)-linoleoyl acyl lipid desaturase.

To obtain additional information on the biosynthesis of conjugated octadecatrienoic acids we decided to continue the analysis of the biosynthesis of calendic acid (8*E*,10*E*,12*Z*-18:3) in the developing seeds of *C. officinalis*. This species is amenable to such studies, because it is easy to grow, its seeds contain large quantities of calendic acid, and most of the studies on the biosynthesis of conjugated octadecatrienoic acids have been performed with seeds from this plant. Here, we describe the cloning of a new type of (1,4)-acyl lipid desaturase, a (8,11)-linoleoyl desaturase that catalyzes the formation of a conjugated triene, calendic acid.

2. Materials and methods

C. officinalis was grown from seeds in a greenhouse under 16 h of artificially supplemented illumination at a temperature ranging between 22 and 30°C. For all experiments developing seeds at a fully expanded green stage were used.

For RNA isolation 20 g of plant material was ground in liquid nitrogen, 100 ml of extraction buffer I (100 mM Tris-HCl, pH 7.5, 25 mM EDTA, 2% (w/v) laurylsarcosyl, 4 M guanidinium thiocyanate, 5% (w/v) PVPP, 1% (v/v) β-mercaptoethanol) was added and the homogenate was shaken for 15 min. After centrifugation at 4000×*g* for 15 min the floating solid lipid phase was discarded and the remaining liquid phase was extracted twice for 15 min with PCI (phenol:chloroform:isoamyl alcohol, 25:24:1). After centrifugation at 4000×*g* for 15 min the upper hydrophilic phase was loaded on a CsCl cushion (5 M CsCl) of 8 ml and centrifuged at 18°C and 100 000×*g* for 18 h. After discarding the supernatant the RNA precipitate was dried, washed with 70% ethanol and extracted for 15 min with a mixture consisting of 7.5 ml of extraction buffer II (100 mM Tris-HCl, pH 8.8, 100 mM NaCl, 5 mM EDTA, 2% (w/v) SDS) and 10 ml PCI. After centrifugation at 4000×*g* for 15 min the upper hydrophilic phase was washed with chloroform and the remaining RNA was precipitated overnight at 4°C by adding an equal volume of 5 M LiCl. After centrifugation for 60 min at 12 000×*g* at 4°C the precipitate was washed twice with 70% ethanol, dried and dissolved in 500 μl water. From this total RNA fraction mRNA was isolated using the Poly-Attract-Kit (Promega, Mannheim, Germany).

Single stranded cDNA (ss-cDNA) was isolated from mRNA of developing marigold seeds by reversed transcription with Superscript-II (Gibco BRL, Eggenstein, Germany). This ss-cDNA was used as template for PCR-based cloning. A 470 bp PCR fragment was amplified with the degenerate sense primer A 5′-CCD TAY TTC TCI TGG AAR WWH AGY CAY CG-3′ and antisense primer B 5′-CCA RTY CCA YTC IGW BGA RTC RTA RTG-3′ derived from the amino acid sequences PYFSWK(Y/I)SHR and HYDS(S/T)EW-(D/N)W, respectively. The PCR reaction was carried out with TfI-

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number AJ245938.
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DNA-Polymerase (Biozym, Hess. Oldendorf, Germany) using an amplification program of 2 min denaturation at 94°C, followed by 10 cycles of 30 s at 94°C, 45 s at 50°C, 1 min at 72°C, followed by 20 cycles of 30 s at 94°C, 45 s at 50°C, 1 min at 72°C (time increment 5 s) and terminated by 2 min extension at 72°C. PCR products of the expected length were cloned in pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced. The fragment CoDes2.2 was chosen for the isolation of a full-length cDNA clone using the Marathon cDNA amplification kit (Clontech, Heidelberg, Germany). To amplify the 5′- and 3′-ends of CoDes2.2 by PCR specific primers were used: 5′-race: primer C 5′-GTG AGG GAG TGA GAG ATG GGT GTG GTG C-3′, primer D 5′-AAC ACA CTT ACA CCT AGT ACT GGA ATT-3′; 3′-race: primer E 5′-TAT TCC AAA CTT CTT AAC AAT CCA CCC G-3′, primer F 5′-CAA TTC CAG TAC TAG GTG TAA GTG TGT T-3′. The PCR reaction was carried out according to the supplier's instructions. The fragments were cloned in pCR2.1-TOPO and sequenced. To obtain the full-length cDNA clone by PCR, specific primers of the expected open reading frame of the entire cDNA of 1.2 kb were used for amplification: sense primer G 5′-ATT AGA GCT CAT GGG TGC TGG TGG TCG GAT GTC G-3′ and antisense primer H 5′-ATT ACT CGA GTG ACA TAC ACC TTT TTG ATT ACA TCT TG-3′. The PCR reaction was carried out with the Expand High Fidelity System (Boehringer, Mannheim, Germany) using an amplification program of 2 min denaturation at 94°C, followed by 10 cycles of 30 s at 94°C, 35 s at 63°C, 2 min at 72°C, followed by 15 cycles of 30 s at 94°C, 35 s at 63°C, 1 min at 72°C (time increment 5 s) and terminated by 2 min extension at 72°C. The fragment was cloned into pGEM-T and the resulting plasmid pCoDes8.11 was sequenced. For expression in *Sacharomyces cerevisiae* the open reading frame of pCoDes8.11 was cloned as a *Sac*I/*Xho*I fragment behind the galactose-inducible promoter *GAL1* into the shuttle vector pYES2 (Invitrogen, Carlsbad, CA, USA) to yield plasmid pYESDes8.11. Plasmid pYESDes8.11 was transformed into the yeast strain INVSC1 (Invitrogen, Carlsbad, CA, USA) by the polyethylene glycol method [10]. The expression of cells harboring plasmid pYESDes8.11 was performed essentially as described before including the exogenous application of linoleic acid [11]. Cells reaching the stationary phase were washed with minimal medium, harvested by centrifugation and stored at −20°C until they were analyzed.

For analysis of the calendic acid content of transformed yeast cells, the cell pellets were disrupted using a sonifier tip and lipids were extracted with hexane/isopropanol according to [12]. After recovery of the organic phase, solvents were evaporated by vacuum and the lipids were reconstituted in methanol, then either directly analyzed by HPLC or subjected to alkaline hydrolysis as described [13]. HPLC analysis of the free fatty acid derivatives was carried out by RP-HPLC as described before [13]. Calendic acid eluted with an retention time of 11.8 min exhibiting the characteristic conjutriene UV spectrum with a maximum at 268 nm [14]. Linoleic acid eluted with a retention time of 12.7 min exhibiting maximal absorbance at 202 nm. Co-injections with authentic standards were performed. The standard of calendic acid was prepared from the triacylglycerol fraction of lipid extracts from marigold seeds by alkaline hydrolysis. Alternatively, the corresponding fatty acid methyl esters were analyzed by gas chromatography/mass spectrometry (GC/MS). For derivation the free fatty acid derivatives were incubated with diazomethane. GC/MS analysis was performed with a Finigan GCQ GC/MS system equipped with a capillary Rtx-5 column (5% diphenyl-95% polydimethyl siloxane, 30 m×0.25 mm; 0.25 μm coating thickness; Restek, Germany). Helium was used as carrier gas (40 cm/s). An electron energy of 70 eV, an ion source temperature of 150°C, and a temperature of 275°C for the transfer line were used. The samples were measured in the EI mode, and the splitless injection mode (opened after 1 min) with an injector temperature of 250°C. The temperature gradient was 60°C–110°C at 25°C/min, 110°C for 1 min, 110°C–270°C at 10°C/min, and 270°C for 10 min. Calendic acid eluted with a retention time of 17.16 min, linoleic acid eluted with a retention time of 15.92 min, and α-linolenic acid eluted with a retention time of 15.97 min.

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CoDES8,11  MGAGGRMSDPSEGK----NILERVPVDPP-FTLSDLKKAIPTHCFERSVIRSSYYVVHDL 55
CaAce      MGGGGRGR--TSQK----PLMERVSVDPP-FTVSDLKQAIPPHCFKRSVIRSSYYIVHDA 53
CpEpox     MGAGGRGR--TSEK----SVMERVSVDPVTFSLSELKQAIPPHCFQRSVIRSSYYVVQDL 54
BoDES12    MGGGGRMPVPTKGKKSKSDVFQRVPSEKPPFTVGDLKKVIPPHCFQRSVLHSFSYVVYDL 60

CoDES8,11  IVAYVFYYLANTYIPLIPTPLAYLAWPVYWFCQASILTGLWVIGHECGHHAFSDYQLIDD 115
CaAce      IIAYIFYFLADKYIPILPAPLAYLAWPLYWFCQASILTGLWVIGHECGHHAFSDYQWVDD 113
CpEpox     IIAYIFYFLANTYIPTLPTSLAYLAWPVYWFCQASVLTGLWILGHECGHHAFSNYTWFDD 114
BoDES12    VIAALFFYTASRYIHLQPHPLSYVAWPLYWFCQGSVLTGVWVIAHECGHHAFSDYQWLDD 120

CoDES8,11  IVGFVLHSALLTPYFSWKYSHRNHHANTNSLDNDEVYIPKRKSKVKIYSKLLNNPPGRVF 175
CaAce      TVGFILHSFLMTPYFSWKYSHRNHHANTNSLDNDEVYIPKSKAKVALYYKVLNHPPGRLL 173
CpEpox     TVGFILHSFLLTPYFSWKFSHRNHHSNTSSIDNDEVYIPKSKSKLARIYKLLNNPPGRLL 174
BoDES12    TVGLLLHSALLVPYFSWKYSHRPHHSNTGSLERDEVFVPKKRSGISWSSEYLNNPPGRVL 180

CoDES8,11  TLVFRLTLGFPLYLLTNISGKKYGRFANHFDPMSPIFNDRERVQVLLSDFGLLAVFYAIK 235
CaAce      IMFITFTLGFPLYLFTNISGKKYERFANHFDPMSPIFKERERFQVLLSDLGLLAVLYGVK 233
CpEpox     VLIIMFTLGFPLYLLTNISGKKYDRFANHFDPMSPIFKERERFQVFLSDLGLLAVFYGIK 234
BoDES12    VLLVQLTLGWPLYLMFNVSGRPYDRFACHFDPKSPIYNDRERLQIYISDAGIVAVMYGLY 240

CoDES8,11  LLVAAKGAAWVINMYAIPVLGVSVFFVLITYLHHTHLSLPHYDSTEWNWIKGALSTIDRD 295
CaAce      LAVAAKGAAWVTCIYGIPVLGVFIFFDIITYLHHTHLSLPHYDSSEWNWLRGALSTIDRD 293
CpEpox     VAVANKGAAWVACMYGVPVLGVFTFFDVITFLHHTHQSSPHYDSTEWNWIRGALSAIDRD 294
BoDES12    RLVAAKGVAWVVCYYGVPLLVVNGFLVLITYLQHTQPSLPHYDSSEWDWLKGALATVDRD 300

CoDES8,11  FGFLNRVFHDVTHTHVLHHLISYIPHYHAKEARDAIKPVLGEYYKIDRTPIFKAMYREAK 355
CaAce      FGFLNSVLHDVTHTHVMHHLFSYIPHYHAKEARDAINTVLGDFYKIDRTPILKAMWREAK 353
CpEpox     FGFLNSVFHDVTHTHVMHHLFSYIPHYHAKEARDAIKPILGDFYMIDRTPILKAMWREGR 354
BoDES12    YGFLNKVLHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGDYYQCDRTPVFKAMYREVK 360

CoDES8,11  ECIYIEPDEDSEHKGVFWY-HKM 377
CaAce      ECIFIEPEKGRESKGVYWY-NKF 375
CpEpox     ECMYIEPDS--KLKGVYWY-HKL 374
BoDES12    ECIYVEADEGDNKKGVFWYKNKL 383
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Fig. 1. Sequence alignment of the (8,11)-linoleoyl desaturase (CoDes8,11) from marigold with the Δ^{12} fatty acid acetylenase from *Crepis alpina* (CaAce, accession number: 081931), the Δ^{12} fatty acid epoxygenase from *Crepis palaestina* (CpEpox, accession number: 065771) and the Δ^{12} acyl lipid desaturase from borage (BoDes12, accession number: 082729). The three characteristic and highly conserved histidine regions are indicated by boxes, and identical amino acids are marked as bold letters.

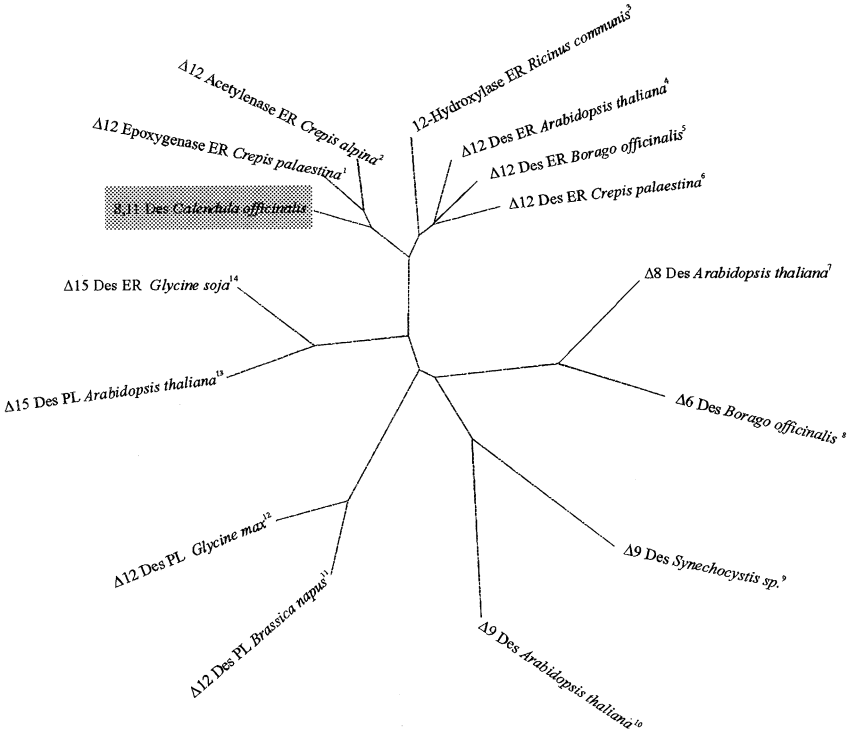

Fig. 2. Phylogenetic tree analysis of plant acyl lipid desaturases. Alignments were generated by the CLUSTAL-X program, and the phylogenetic tree was made with 'TreeView'. [1]Y16283, [2]Y16285, [3]U22378, [4]L26296, [5]O82729, [6]Y16284, [7]AJ224161, [8]U79010, [9]D16547, [10]D88536, [11]P48627, [12]P48628, [13]L22961, [14]L22964.

3. Results

3.1. PCR-based cloning and isolation of a full-length cDNA

For PCR-based cloning degenerate primers, deduced from conserved regions of acyl lipid desaturases, were synthesized according to [11,15,16]. The template used was ss-cDNA from *C. officinalis*, which was reverse-transcribed from mRNA of developing seeds at a fully expanded green stage. PCR products of the expected length were cloned and sequenced. Data base searches and alignments with these fragments indicated similarities to plant acyl lipid desaturases with different regio specificities. Except for one (CD2.2), all were classified either as putative members of the classical Δ^{12} desaturases or as putative sphingolipid desaturases based on identities more than 75% [16]. However, the 470 bp fragment CD2.2 exhibited highest identities to the Δ^{12} fatty acid acetylenase from *Crepis alpina* and the Δ^{12} fatty acid epoxygenase from *Crepis palaestina* [15]. To isolate the full-length cDNA clone, PCR with specific primers was used to amplify the 5′- and 3′-ends of CD2.2. The fragments were cloned and sequenced. With specific primers for the expected open reading frame the entire cDNA of 1.2 kb was amplified by PCR and cloned into pGEM-T. The resulting fragment CoDes8.11 was sequenced. The full-length cDNA had a length of 1131 bp encoding a protein of 377 amino acids with a calculated molecular mass of 43.6 kDa. This desaturase sequence showed highest identities with the Δ^{12} acetylenase from *C. alpina* (74%) [15], the Δ^{12} epoxygenase from *C. palaestina* (73%) [15], and the Δ^{12} desaturase from borage (62%) [17] (Fig. 1). The three characteristic and highly conserved histidine regions of the active site of the Δ^{12} acyl lipid desaturases are indicated by boxes. These findings were further supported by phylogenetic analysis according to [17]. As shown in Fig. 2, the newly identified (8,11)-linoleoyl desaturase groups with the Δ^{12} acyl lipid desaturase family. However, it forms one subfamily with the Δ^{12} acetylenase and the Δ^{12} epoxygenase. All members of this subfamily are clearly separated from the classical Δ^{12} desaturases and 12-hydroxylases.

3.2. Functional expression in S. cerevisiae and fatty acid analysis

In order to elucidate the catalytic properties of this new desaturase, CoDes8.11 was expressed in yeast cells under the control of the galactose-inducible promoter *GAL1*. Since yeast cells do not contain dienoic fatty acids as substrates for the (8,11)-linoleoyl desaturase [8], the expression was performed with supplementation of linoleic acid. Galactose-induced yeast cells were used for the analysis either of fatty acid methyl esters by GC/MS or of free fatty acids by HPLC. The methyl ester of calendic acid was separated from the classical dienoic and trienoic fatty acids by GC/MS. It eluted with a retention time of 17.16 min, whereas the methyl ester of linoleic acid eluted with a retention time of 15.92 min, and that of α-linolenic acid eluted with a retention time of 15.97 min (data not shown). However, using this method calendic acid was only identified by its characteristic retention time, because the corresponding mass spectra of the methyl esters of calendic acid and α-linolenic acid showed no characteristic fragmentation patterns when compared against each other. The spectra differed only in their relative intensities of the obtained fragments (data not shown). Therefore, we decided to analyze the formation of calendic acid within yeast cells overexpressing CoDes8.11 by RP-HPLC, because of the characteristic UV spectrum of calendic acid and the substantial loss of cal-

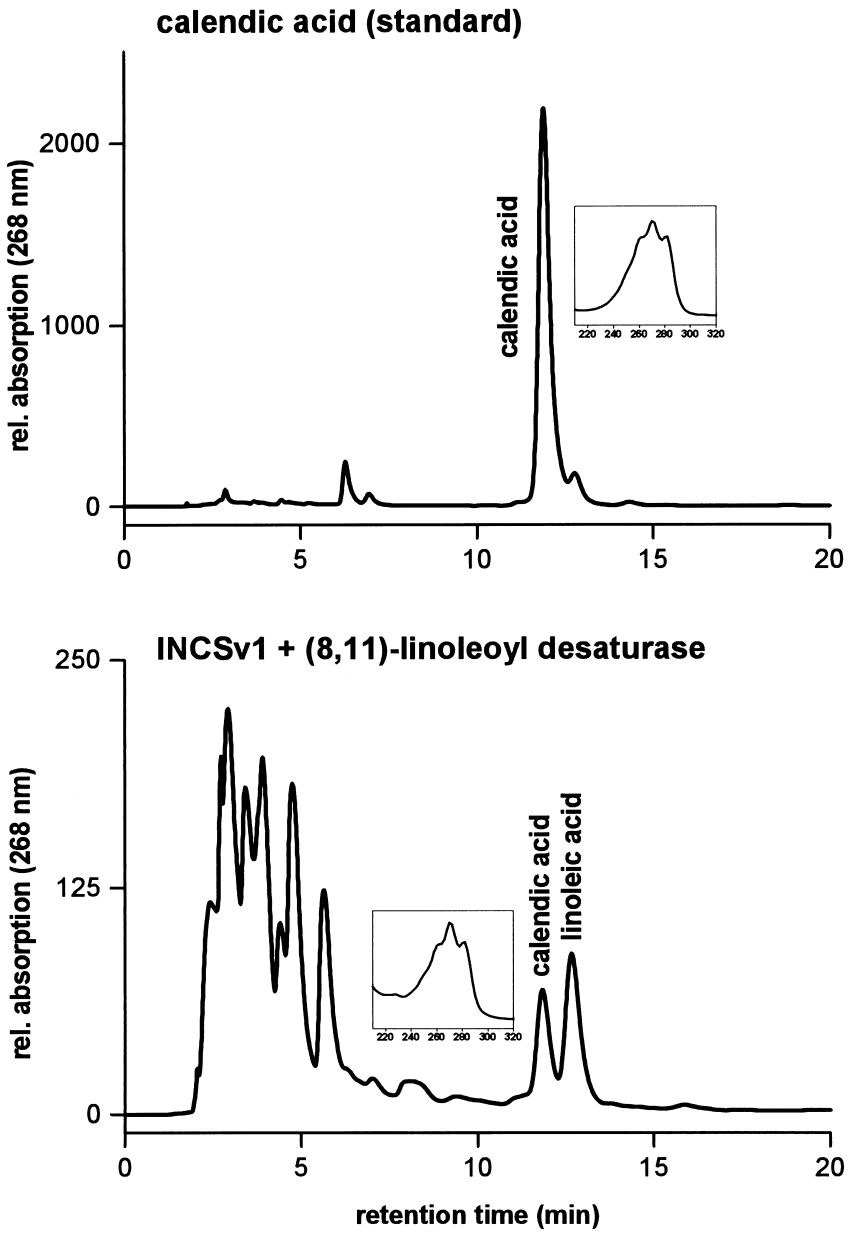

Fig. 3. Reversed-phase HPLC analysis of calendic acid either isolated from the triacylglycerol fraction of marigold seeds or isolated from yeast cells transformed with the marigold (8,11)-linoleoyl desaturase. The lipids were extracted and analyzed by HPLC as described [12]. Calendic acid and linoleic acid were characterized by co-elution of authentic standards. The insets show the UV spectra of calendic acid exhibiting a maximum at 268 nm (maximum for conjugated triene system).

endic acid during derivation into its methyl ester for GC/MS analysis. As a standard calendic acid was isolated from the triacylglycerol fraction of marigold seeds and analyzed by RP-HPLC (Fig. 3, upper panel). The retention time of calendic acid was 11.8 min and as shown in the inset it exhibited a characteristic UV spectrum of conjutrienes with the three relative maxima at 262 nm, 268 nm and 282 nm [14]. Yeast cells harboring only the empty vector synthesized no calendic acid (data not shown). However, *S. cerevisiae* cells expressing the CoDes8.11 cDNA were capable of forming a fatty acid which co-elutes with calendic acid at 11.8 min and exhibits the characteristic UV spectrum of conjutrienes (Fig. 3, lower panel, first peak at 11.8 min). The second peak, eluting close behind calendic acid at 12.7 min, was identified as linoleic acid. This indicates that the newly isolated cDNA encodes a desaturase which is capable of converting linoleic acid into calendic acid upon expression in yeast cells. Moreover, no calendic acid was found in the fraction of free fatty acids (data not shown). This supports the idea that calendic acid formation may occur while the acyl moiety is esterified to phosphatidylcholine, as has been suggested before [9].

4. Discussion

Here, we report the isolation and characterization of a cDNA encoding a new type of desaturase catalyzing a (1,4)-dehydrogenation reaction. The cDNA encoding an (8,11)-linoleoyl desaturase was cloned by a PCR-based approach as has been described before [11,16]. So far, this cDNA could not be identified by EST sequencing of cDNA libraries from

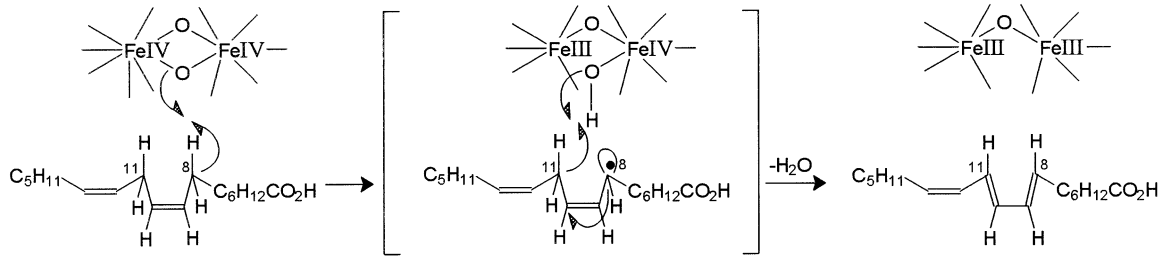

Fig. 4. Speculative mechanism for (8,11)-linoleoyl desaturases (adapted from [20]) based on the proposed catalytic mechanism for the Δ^9 desaturase from castor bean [21]. Not shown is the resting level of the (8,11)-linoleoyl desaturase harboring two diferrous Fe(II) atoms. To reach this level an additional reduction by two more electrons is required.

developing marigold seeds, indicating that the expression of this (8,11)-linoleoyl desaturase gene is either very low or developmentally regulated. The deduced protein shared highest homologies with the recently identified Δ^{12} acetylenase and Δ^{12} epoxygenase from *Crepis* [15]. All three enzymes convert the double bond in the Δ^{12} position of linoleic acid esterified to phosphatidylcholine into groups with new functionalities. In addition, within a phylogenetic analysis these three enzymes could be clearly separated from the other members of the Δ^{12} acyl lipid desaturase family (Fig. 2).

As shown by heterologous expression in yeast cells, the enzyme converts linoleic acid into a conjugated octadecatrienoic acid (Fig. 3). Based on the observation that linoleic acid is the precursor of calendic acid one may propose a new reaction mechanism for this Δ^{12}-type desaturase. Whereas within the classical Δ^{12} acyl lipid desaturases a (1,2)-*syn* dehydrogenation at the carbon atoms C-12 and C-13 of oleic acid may occur [18], one may assume in the case of this new desaturase a (1,4)-dehydrogenation at the carbon atoms C-8 and C-11 of linoleic acid as shown in a speculative reaction scheme in Fig. 4. Within linoleic acid the elimination of protons at these two positions might be sterically favored, because the carbon atoms at the positions C-8 and C-11 are localized near each other in the three-dimensional structure of the fatty acid molecule. For the conversion of linoleic acid into α-eleostearic acid one may assume a homologous enzyme catalyzing a (1,4)-dehydrogenation at the carbon atoms C-14 and C-11 of linoleic acid. Structural determinants which point to this new regio specificity in proton abstraction can hardly be determined by sequence comparison. However, conjugated octadecatrienoic acids are widespread in plant seed oils [19]. Therefore, sequence comparisons between this enzyme and novel yet to be isolated (1,4)-acyl lipid desaturases might help to identify structural characteristics or differences in these enzymes which lead to the observed regio and stereochemical specificities within this subfamily of Δ^{12} desaturases. Even the differences between this (8,11)-linoleoyl desaturase catalyzing the formation of calendic acid (8*E*,10*E*,12*Z*-18:3) and the corresponding (8,11)-linoleoyl desaturase catalyzing the formation of jacaric acid (8*Z*,10*E*,12*Z*-18:3) from *Jacaranda mimosifolia* [1] is an interesting but open question. With the isolation of this enzyme the first example has been given for a (1,4)-dehydrogenation reaction catalyzed by a desaturase on a dienoic fatty acid. However, this reaction has been suggested recently for a slightly different substrate as well, the conversion of a monoenoic fatty acid into a conjudienoic fatty acid within the biosynthesis of bombykol, a sex pheromone of the female moth *Bombyx mori* [20].

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